

Prevalence of *Giardia duodenalis* genotypes in pre-weaned dairy calves

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Accepted 1 July 2004

Abstract

To determine the prevalence of *Giardia* genotypes in pre-weaned dairy calves, fecal samples were collected from a minimum of 18, 1–7-week-old dairy calves per farm on two farms each in the states of Vermont, New York, Pennsylvania, Maryland, Virginia, North Carolina, and Florida. Samples cleaned of fecal debris and concentrated using CsCl density gradient centrifugation were stained and examined by immunofluorescence microscopy and also subjected to PCR and gene sequence analysis. Prevalence by PCR ranged from 9% on a farm in Pennsylvania to 93% on a farm in Vermont, with an average prevalence for 407 calves on 14 farms of 40%. Gene sequence analysis of the TPI, β -giardin and 16S rRNA genes revealed 85% of the positive samples to be Assemblage E, while 15% were Assemblage A, although the percentages of these genotypes varied greatly from farm to farm. Some farms had no Assemblage A *Giardia*. Thus, while a majority of the calves were infected with a genotype that is not known to be infectious for humans, calves on 7 of 14 farms did harbor Assemblage A *Giardia*. Calves should be considered as a potential source of human infectious cysts in the environment.

Published by Elsevier B.V.

Keywords: *Giardia* spp; Prevalence; Calves; PCR; Genotype

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1. Introduction

Giardia duodenalis (syn. *G. lamblia*, *G. intestinalis*) is a commonly identified intestinal parasite of mammals, including humans. Historically, giardiasis has been the most commonly diagnosed waterborne disease in developed countries (Levine et al., 1990), and *Giardia* spp. cysts are routinely detected in surface waters. However, the relative contributions of the organisms from different sources are not well known. One complicating factor is that genetic and biological differences within the *G. duodenalis* complex have been masked by the fact that subtypes are morphologically indistinguishable (Thompson et al., 2000). Thus, it appears that not all organisms classified as *G. duodenalis* have the same biological potential to pose a risk of human infection. Molecular characterization has proven a useful tool for identifying genotypes or assemblages, some of which appear to have restricted host ranges (Monis et al., 1999, 2003). Assemblages A and B have the widest host ranges, encompassing humans and a variety of other animals; Assemblage A has also been reported in cattle (O'Handley et al., 2000; van Keulen et al., 2002; Appelbee et al., 2003). Assemblages C and D have been reported only in dogs, Assemblage E has been reported only in hoofed livestock, Assemblage F has been reported only in cats, and Assemblage G has been reported only in rats (Monis et al., 2003).

Although prevalence data on *Giardia* infection in cattle often vary markedly, many point prevalence studies of cattle report a significant percentage of animals infected with *Giardia*, and cumulative prevalence often reaches 100% (Olson et al., 1997a, 1997b; Ruest et al., 1998; O'Handley et al., 1999; Ralston et al., 2003). However, only a few studies have reported the *Giardia* genotypes that are present in infected cattle. Surveys in Canada have generally indicated that Assemblage E is the predominant genotype in cattle, but Assemblage A has also been reported (O'Handley et al., 2000; Appelbee et al., 2003). Analysis of three bovine samples from the state of New York indicated the presence of Assemblage A in cattle in the US as well (van Keulen et al., 2002).

To our knowledge, there have been no multi-state prevalence studies for *Giardia* conducted in the US that include genotypic analysis of isolates. Thus, the extent to which *Giardia* infected cattle in the US might pose a risk of human infection is unknown. Certainly if cattle can harbor Assemblage A *Giardia duodenalis*, they must be considered a potential source of human infective cysts in the environment.

The current study was conducted to assess the prevalence of *Giardia* in pre-weaned dairy calves along the eastern US and to identify the genotypes that were present.

2. Materials and methods

2.1. Farms

Two commercial dairy farms were selected in each of the following states: Vermont, New York, Pennsylvania, Maryland, Virginia, North Carolina, and Florida. The only selection criterion was the ability to provide a minimum of 15 calves of the appropriate age for sampling.

2.2. Calves

Calves (both males and females) between the ages of 1 and 7 weeks were randomly selected for sampling on each farm. The number of animals from which useful specimens were obtained ranged from 18 to 61 per farm (Table 1).

2.3. Fecal sample collection and processing

Approximately 50 g of feces were collected directly from the rectum of each calf. Samples were collected into plastic screw cap specimen cups and placed on ice in an insulated container for transport back to Beltsville, Maryland, where they were processed within 4 days of collection. Due to the age of the calves, it was not always possible to collect 50 g of fecal material, in cases where only a small amount of feces could be obtained, direct fecal smears were prepared on glass microscope slides and stained as described below. Processing of larger fecal volumes was as follows. Fifteen grams of feces were placed into a 50 ml tube and thoroughly mixed with 35 ml dH₂O. The fecal suspension was passed through a 45 µm screen, collected into a second 50 ml tube and adjusted to a final volume of 50 ml with dH₂O. The tubes were centrifuged at 1500 × g for 15 min, the supernatant discarded and the fecal pellet re-suspended in 25 ml dH₂O. Twenty-five milliliter of CsCl (1.4 g/ml) was added to each tube and the samples were thoroughly mixed. The samples were subjected to a second

Table 1

Farm designations, number of calves sampled on each farm, and prevalence of *Giardia duodenalis* and genotypes in pre-weaned dairy calves on 14 farms in seven east coast states

State	Farm	Number of animals sampled	Number positive by IFA	Number of samples analyzed by PCR	Number positive by PCR	Assemblage (percent of PCR positive isolates)	
						A	E
Vermont	VT-1	23	13 (57%)	15	14 (93%)	29	71
	VT-2	23	19 (83%)	17	15 (88%)	27	73
New York	NY-1	45	13 (29%)	25	12 (48%)	38	62
	NY-2	42	28 (67%)	36	11 (30%)	45	55
Pennsylvania	PA-1	37	6 (16%)	32	3 (9%)	0	100
	PA-2	43	16 (37%)	24	10 (42%)	40	60
Maryland	MD-1	40	12 (30%)	39	11 (28%)	0	100
	MD-2	40	5 (13%)	37	4 (11%)	25	75
Virginia	VA-1	25	13 (52%)	22	13 (59%)	8	92
	VA-2	18	11 (61%)	12	9 (75%)	0	100
North Carolina	NC-1	38	25 (66%)	33	20 (61%)	0	100
	NC-2	61	40 (66%)	49	23 (47%)	0	100
Florida	FL-1	50	14 (28%)	40	11 (28%)	0	100
	FL-2	28	9 (32%)	26	8 (31%)	0	100
Total		513	224 (44%)	407	164 (40%)	15	85

centrifugation at $250 \times g$ for 20 min. Following the second centrifugation, the top 4 ml of supernatant was aspirated from each sample and transferred into a 15 ml tube. De-ionized H_2O was added to all tubes to bring the final volume to 15 ml. Samples were washed twice with dH_2O and the final pellet was suspended in 500 μ l of dH_2O . Portions of this 500 μ l suspension were used for immunofluorescence analysis or molecular analysis as described below. Samples from which direct smears were prepared could not be subjected to molecular analysis due to insufficient sample material.

2.4. Immunofluorescent analysis

From each fecal suspension, a 100 μ l aliquot was transferred to a microcentrifuge tube and washed once with dH_2O . The pellet was suspended in 25 μ l of premixed Mer/Fluor reagents (Meridian Diagnostics, Cincinnati, OH). Premixed Mer/Fluor reagents were prepared as follows: combine Mer/Fluor test reagent and counterstain, add 3 ml sterile PBS and mix well. Two microliter of the fecal/stain suspension was transferred to one well of a three-well slide (Cel-line, HTC, Portsmouth, NH); a coverslip was placed on the slide and slides were examined at $400\times$ using a Zeiss Axioskop microscope equipped with epifluorescence and an FITC-Texas Red dual wavelength filter. Slides with direct fecal smears were stained by covering the sample with 100 μ l of premixed Mer/Fluor reagent and incubating at room temperature for 30 min. Each slide was rinsed with dH_2O , coverslipped and examined as described above.

2.5. DNA extraction

Total DNA was extracted from each CsCl-cleaned fecal sample using a DNeasyTissue Kit (Qiagen, Valencia, CA) with a slightly modified protocol. The protocol, described below, utilized reagents provided by the manufacturer. A total of 50 μ l of processed feces were suspended in 180 μ l of ATL buffer and thoroughly mixed by vortexing. To this suspension, 20 μ l of proteinase K (20 mg/ml) was added, and the sample was thoroughly mixed. Following an overnight incubation of the mixture at $55^\circ C$, 200 μ l of AL buffer was added. The remaining protocol followed manufacturer's instructions with one exception. To increase the quantity of recovered DNA, the nucleic acid was eluted in 100 μ l of AE buffer.

2.6. Amplification of gene segments by polymerase chain reaction

Fragments of the *ssrRNA* (~ 292 bp), β -giardin (~ 750 bp), and *TPI* (~ 500 bp) genes were amplified by PCR as previously described (Hopkins et al., 1997; Cacciò et al., 2002; Sulaiman et al., 2003). PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining.

2.7. DNA sequence analysis

PCR products were purified using EXO-SAP enzyme (USB Corporation, Cleveland, OH). Purified products were sequenced with the same PCR primers used for the original

amplification in 10 µl reactions, Big Dye™ chemistries, and an ABI3100 sequencer analyzer (Applied Biosystems, Foster City, CA). Each sample was sequenced in both directions. Sequence chromatograms from each strand were aligned and inspected using Lasergene software (DNASTAR, Inc., Madison, WI). All of the PCR positive samples were sequenced. To determine the *Giardia* genotype of in the samples, each sequence for all three gene fragments was independently compared to GenBank sequences of *Giardia* genotypes.

3. Results

The number and location of calves infected with *Giardia* as determined by IFA and PCR are shown in Table 1. The differences in total numbers of samples analyzed between these two methods reflects the fact that when direct fecal smears were prepared as described above, there was insufficient feces available for molecular analysis. Thus, analysis of different numbers of samples by IFA and PCR, likely explains, at least in part, the differences in results reported for these two techniques. Of 513 calves on 14 farms examined by IFA, 224 (44%) were *Giardia* positive, and of 407 samples examined by PCR, 164 (40%) were *Giardia* positive. The prevalence of *Giardia* infection varied considerably between farms, with the lowest prevalence (9%) on PA-1 and the highest prevalence (93%) on VT-1. Overall, on 7 and 5 of the 14 farms greater than 50% of the calves were found IFA or PCR positive for *Giardia*, respectively. When examined by age, the percentage of calves infected with *Giardia* increased with the age of the calves, reaching a plateau around 60% by 5 weeks of age (Fig. 1).

The percentages of *Giardia duodenalis* genotypes found on the farms are presented in Table 1. Two genotypes were identified: Assemblage E, which has been reported to infect

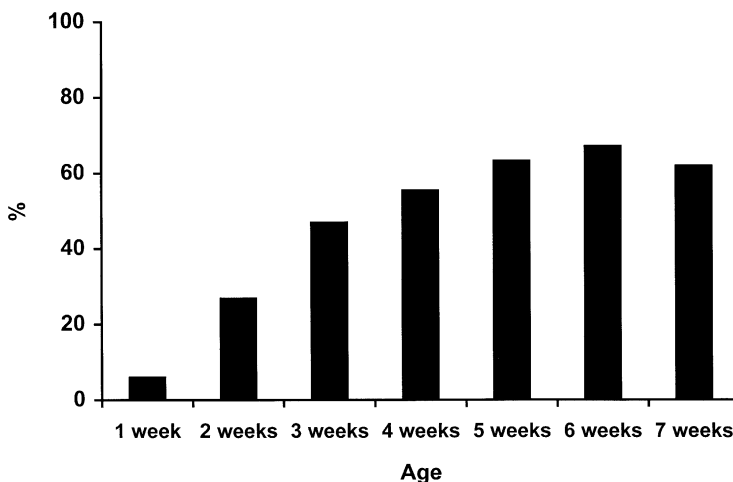


Fig. 1. Age related prevalence of *Giardia duodenalis* in pre-weaned dairy calves as determined by IFA. Data was averaged across 14 farms in seven east coast states.

only hoofed-livestock, and Assemblage A, which is infectious for humans and a number of other mammals. Across all farms, 15% of the *Giardia* positive animals were infected with Assemblage A, while 85% were infected with Assemblage E. Assemblage A was found on 7 of 14 farms: four of four farms in the northeast, three of six farms in the mid-Atlantic, and zero of four farms in the south. On farms where Assemblage A was present, this assemblage represented, on average, 30% of the *Giardia* isolated, with the lowest percentage on VA-1 (8%) and the highest percentage on NY-2 (45%).

4. Discussion

Giardia infection has been reported frequently in young calves. While point prevalence studies often report widely varying levels of infection, the cumulative prevalence for a given farm has been reported to be 100% (Xiao and Herd, 1994; O'Handley et al., 1999). Because cyst excretion can be intermittent (Buret et al., 1990), and only a single fecal sample was collected in the present study, it is likely that the point prevalence data presented herein underestimates the actual numbers of infected animals on a given farm. *Giardia duodenalis* cysts were detected in the feces of dairy calves on all 14 farms examined, with prevalence by IFA and PCR ranging from 13 to 83% and 9 to 93%, respectively. PCR was generally more sensitive than IFA, often detecting a higher number of positive samples. On some occasions, however, IFA detected a greater number of positive samples. This could be due in part to the fact that PCR analysis could not be conducted when there was only sufficient fecal material to prepare a direct smear for IFA. Previous point prevalence studies in North America have also reported a wide range in the number of *Giardia* infected animals (Xiao, 1994; Xiao and Herd, 1994; Olson et al., 1997a, 1997b; O'Handley et al., 1999, 2000). One previous study (Huetink et al., 2001) indicated that the prevalence of *Giardia* infection reached the highest levels at 4–5 months of age, but was much lower in calves 1–2 months of age. Another study (O'Handley et al., 1999) reported a prevalence of about 25% at 1 month of age and a 60–80% prevalence in calves aged 2–3 months. In the current study, the prevalence of infection reached 25% in 2-week-old calves, approached 50% in 3-week-old calves, and peaked around 60% in 6–7 week-old calves. It is possible that these differences are due to variations in assay methodology and/or environmental factors.

The prevalence of *Giardia* genotypes was determined by DNA sequence analysis of three genes (TPI, β -giardin, 16S rRNA) for every PCR positive sample. Thus, sequence data were obtained for all 164 samples. This is the first known prevalence study to obtain sequence information on all PCR positive samples. There was complete agreement on sample genotype between the three gene sequences. Nucleotide sequences of the TPI, 16S rRNA, and β -giardin genes of *G. duodenalis* isolates from calves in the current study, representing Assemblages A and E were deposited in GenBank under accession nos. [AY655700–AY655706](#). Assemblage A *Giardia* was detected at varying levels in calves on 7 of the 14 farms. Assemblage E *Giardia*, with a host range limited to hoofed-livestock, was detected in calves on all farms with seven farms having exclusively Assemblage E. On farms where Assemblage A was detected, the prevalence ranged from 8% (VA-1) to 45% (NY-2) of the isolates. Thus, while Assemblage A represented 15% of the isolates in the

study as a whole, on 7 of 14 farms, Assemblage A represented 30% of the positive isolates. Previous studies using sequence analysis have reported the prevalence of Assemblage A as 20% or less in dairy calves (O'Handley et al., 2000) and only about 2% in beef calves (Appelbee et al., 2003); however, in these studies, genotype was determined only on a subset of the *Giardia* positive samples. Whereas in the current study, genotypic analysis was performed on all of the samples that were PCR positive for *Giardia*.

Interestingly, in the current study, all four farms in the northeastern states were positive for Assemblage A, an average of 35% of the 52 *Giardia* isolates. In the mid-Atlantic states, three of six farms were positive for Assemblage A, an average of 12% of 50 *Giardia* isolates. In the southeastern states, however, none of the 62 *Giardia* isolates obtained from four farms was Assemblage A. Due to the small number of farms (2) per state, it is unclear if these results truly represent differences in the geographical distribution of Assemblage A, however, they are noteworthy. It will be necessary to obtain additional genotype data from farms both within and outside of these regions to determine if indeed there are regional or geographic differences.

The presence of Assemblage A on 50% of the farms and its high prevalence on several farms indicate that calves can be a source *G. duodenalis* cysts that have the potential to infect humans. Especially, considering that some of the highest levels of Assemblage A were found in areas of large human populations centers. Certainly the higher prevalence of Assemblage A in areas of greater human populations raises the possibility these organisms are being transmitted back and forth between animals and humans, thus maintaining higher levels of this genotype.

Although the present study is the most extensive prevalence study of *Giardia* in the US utilizing genotype determination, it still represents only a small number of farms over a wide area. Clearly, additional data on *Giardia* genotypes in cattle is needed from watershed areas surrounding major metropolitan centers. This information will add to our ability to estimate the risk of giardiasis in humans from cattle.

Acknowledgements

The authors wish to thank Kristie Ludwig, Robert Palmer, and Courtney Anderson for their expert logistical and technical support.

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